Virology 380 (2008) 26–33

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Phosphorylation of human respiratory syncytial virus P protein at serine 54 regulates viral uncoating

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ARTICLE INFO

Article history:
Received 8 April 2008
Returned to author for revision 19 May 2008
Accepted 27 June 2008
Available online 15 August 2008

Keywords:
Human respiratory syncytial virus (HRSV)
P protein
Phosphorylation
Uncoating
Lithium-sensitive kinase
Inhibitors
PP2A phosphatase
Prevention of HRSV infections

ABSTRACT

The human respiratory syncytial virus (HRSV) structural P protein, phosphorylated at serine (S) and threonine (T) residues, is a co-factor of viral RNA polymerase. The phosphorylation of S54 is controlled by the coordinated action of two cellular enzymes: a lithium-sensitive kinase, probably glycogen synthetase kinase (GSK-3) β and protein phosphatase 2A (PP2A).

Inhibition of lithium-sensitive kinase, soon after infection, blocks the viral growth cycle by inhibiting synthesis and/or accumulation of viral RNAs, proteins and extracellular particles. P protein phosphorylation at S54 is required to liberate viral ribonucleoproteins (RNPs) from M protein, during the uncoating process. Kinase inhibition, late in infection, produces a decrease in genomic RNA and infectious viral particles.

LiCl, intranasally applied to mice infected with HRSV A2 strain, reduces the number of mice with virus in their lungs and the virus titre. Administration of LiCl to humans via aerosol should prevent HRSV infection, without secondary effects.

Introduction

Suitable tools to control HRSV infections are: vaccines (Whitehead et al., 1999), antiviral compounds (Pastey et al., 2000;Bitko and Barik, 2001), and neutralizing monoclonal antibodies (Zhao and Sullender, 2005; Wu et al., 2008; Boivin et al., 2008). This diversity responds to the different physiological characteristics of human populations (babies and toddlers, elderly and immunocompromised adults) mainly affected by lower tract respiratory infections, caused by this pneumovirus of the *Paramyxoviridae* family (Hall, 2001).

To develop specific antiviral compounds is important to understand how viral proteins work during the viral growth cycle, particularly those involved in distinct processes of viral metabolism, like viral RNA synthesis and extracellular particle formation. Those viral proteins are ideal targets for antiviral compounds.

HRSV has a single strand RNA, of 15,222 nucleotides in length, as its genome, that encodes 10 genes, in the following order 3′NS1-NS2-N-P-M-SH-G-F-M2-L-5′. These genes express the corresponding viral proteins, except for the M2 gene that expresses M2-1 and M2-2 proteins. The viral RNA is enwrapped by N protein in the helical nucleocapsid (NC). To NC, L, P and M2-1 proteins are bound forming the ribonucleoprotein complex (RNP). This complex is the inner part of the virion, surrounded by the M protein, that connects it to the membranous envelope in which the viral glycoproteins F, G and SH are inserted (Collins and Crowe, 2007).

In the formation of the extracellular viral particles, M protein plays a crucial role. It is included in membranous vesicles, when expressed in the absence of the rest of the viral proteins (Asenjo et al., 2005), in agreement with its role as the main determinant of enveloped viral particle formation (Teng and Collins, 1998).

The structural P phosphoprotein is an essential co-factor of L protein, the active viral RNA polymerase being the complex P–L. Its role, during transcription and replication, is to locate L protein on the NC template and to maintain N protein in a functional RNA encapsidation state (N0) (Collins and Crowe, 2007). It is phosphorylated at several serine and threonine residues, by addition of phosphate groups with different turnover. In Long strain P protein, phosphate groups with low turnover, are added to S232 and perhaps to S237 (Sanchez-Seco et al., 1995); with intermediate turnover to S116, S117and /119 (Navarro et al., 1991); with high turnover to S30, S39, S42, S45, S54 and perhaps to T46 (Asenjo et al., 2005) and with very high turnover to T108. P protein phosphorylations with low and intermediate turnover are related to the extracellular viral particle formation (Villanueva et al., 1991; Lu et al., 2002; Asenjo et al., 2005; Villanueva et al., 2000). Phosphorylation at T108 regulates the participation of M2-1 protein in viral transcription (Asenjo et al., 2006).

An interaction between M and P proteins, inhibited by P phosphorylation at S54, has been described (Asenjo et al., 2005). The cellular phosphatase PP2A is involved in its control (Asenjo et al.,

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doi:10.1016/j.virol.2008.06.045

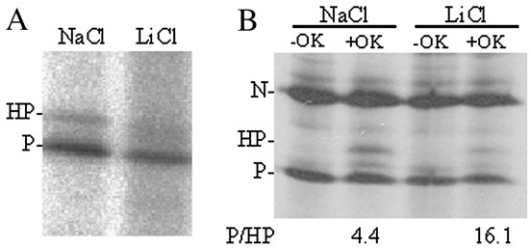

Fig. 1. P protein is phosphorylated at S54 by a lithium sensitive protein kinase. (A) P protein was transiently co-expressed with the small *t* SV40 antigen, labelled with ^{35}S-methione, in the presence of 15 mM LiCl or NaCl, and from the correspoding cellular extracts (Materials and methods) P protein was immunoprecipitated, using P monoclonal antibody RS1/P. The immunoprecipitated proteins were fractionated by SDS-PAGE and visualised by auto-radiography (Asenjo et al., 2006). (B) HRSV infected HEp-2 cells, between 24-30 h post-infection were treated with 15mM LiCl or NaCl and labelled, between 27-30 h, with ^{35}S-methionine, in the presence (+) or in the absence (-) of OKA (Materials and methods). The cellular extracts were obtained and processed as described in (A). The ratio P/HP was determined by densitometry using the pCBAs program.

2005). This P protein residue has the described recognition site of the cellular protein kinase GSK-3 (Moreno et al., 1996).

We resonated that if this cellular kinase is involved in P protein phosphorylation at S54 and the detected P–M interaction (Asenjo et al., 2005) is related to RNP's inclusion into mature viral particles, the inhibition of GSK-3 activity by LiCl salt, a practice widely used in the treatment of bipolar human depression (Cade, 1949; Cade, 1999; Tondo et al., 1997; Manji and Moore, 1999), could be used to limit HRSV infections, by decreasing the RNA synthesis capacity of viral RNPs, due to their premature interaction with M protein. When GSK-3 is active, this interaction is delayed, due to P protein phosphorylation at S54.

The obtained results testing this hypothesis, have indicated that the inhibition of a lithium-sensitive kinase, probably the GSK-3 β isoform, stops the viral growth cycle by blocking virus uncoating, the reverse process of RNP's coating by M protein occurring during extracellular viral particle formation (Lamb and Kolakfosky, 2005). Later post-infection, the inhibition imbalances viral transcription-replication, a phenotype displayed by a recombinant HRSV lacking M2-2 protein (Bermingham and Collins, 1999).

The inhibitory effect of LiCl treatment on HRSV infection, was also observed in animals, without secondary effects. Thus, this treatment applied as an aerosol to the upper respiratory tract in humans, may prevent HRSV infections.

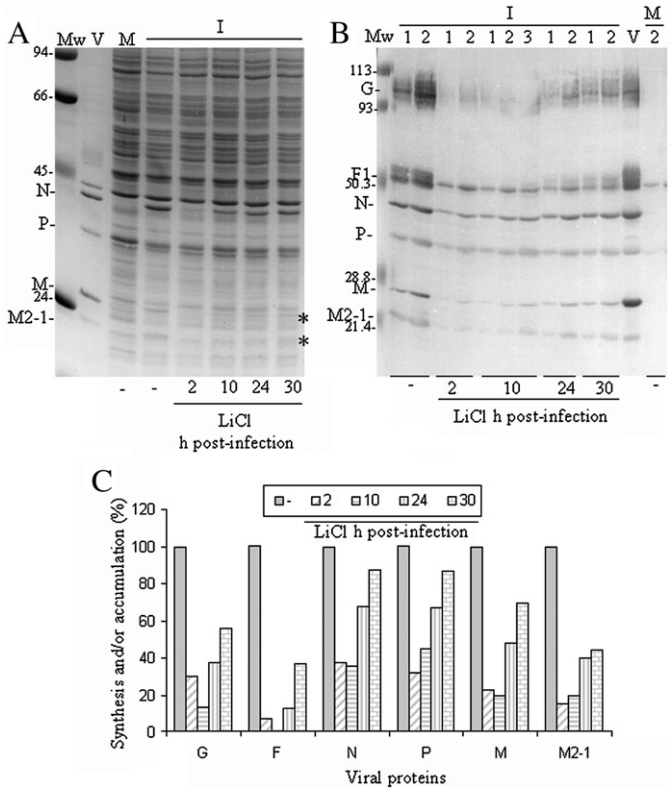

Fig. 2. Effect of lithium addition at different post-infection times, on viral protein synthesis and/or accumulation. (A) Uninfected (lane M) or HRSV-infected (lanes I) HEp-2 cells were untreated (lanes −) or treated with 15 mM LiCl, at the indicated times post-infection (lower part of the panel). Forty eight hours post-infection, the corresponding cellular extracts were obtained (Materials and methods). After quantitation, 20 μg of total protein from the different cellular extracts were analysed by SDS-PAGE and visualised by staining with Coomassie blue. (B) Different total protein amounts (lanes 1, 2 and 3 correspond to 12.5, 25 and 50 μg, respectively) from the different cellular extracts were separated by SDS-PAGE and analysed by Western blot with an anti-HRSV serum. In panels A and B, left part, the electrophoretical mobilities of MW markers (lane MW) and those of proteins contained in purified extracellular viral particles (lane V) are indicated. (C) The results shown in panel B, representative of those obtained in several experiments, were quantified by densitometric analysis using the pCBas program. The obtained values for the different viral proteins are expressed as percentages of the values obtained in the absence of LiCl that were considered as 100%.

Results

A cellular lithium-sensitive protein kinase is involved in P protein phosphorylation at S54. Two phosphorylated serines in HRSV P protein, those at positions 30 and 54, have the recognition consensus sequence described for the kinase GSK-3 (Moreno et al., 1996) (SP or SXXXS*, being S* in phosphorylated form and S the target residue to be phosphorylated by GSK-3).

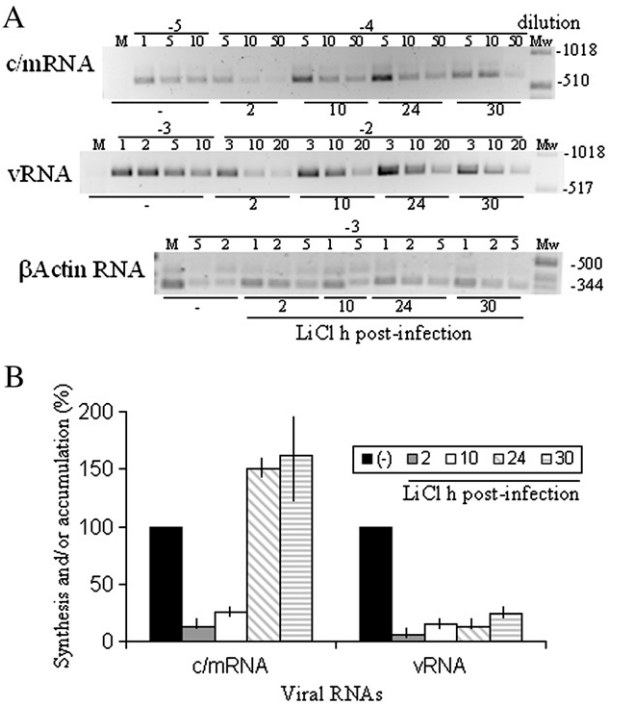

Fig. 3. Effect of lithium addition, at different post-infection times, on viral RNAs synthesis and/or accumulation. Panel A, half of the cultures analysed in Fig. 2 were processed to obtain total RNA by Trizol method following the supplier's instructions. Dilutions of these materials were used to quantify (indicated as log to base 10, in the upper part), by semiquantitative RT-PCR, the amounts of cRNA plus mRNA and vRNA, with oligonucleotides corresponding to genes P and M2, respectively. As an internal control, β-actin mRNA was quantified. The amplified DNA fragments were analysed by 1% agarose gel electrophoresis in TBE buffer, visualised by staining with ethidium bromide and quantified by the pCBAS program (see details in Materials and methods). The average results of six experiments are shown in panel B, expressed as percentage of values determined in the absence of LiCl. Value ranges are indicated.

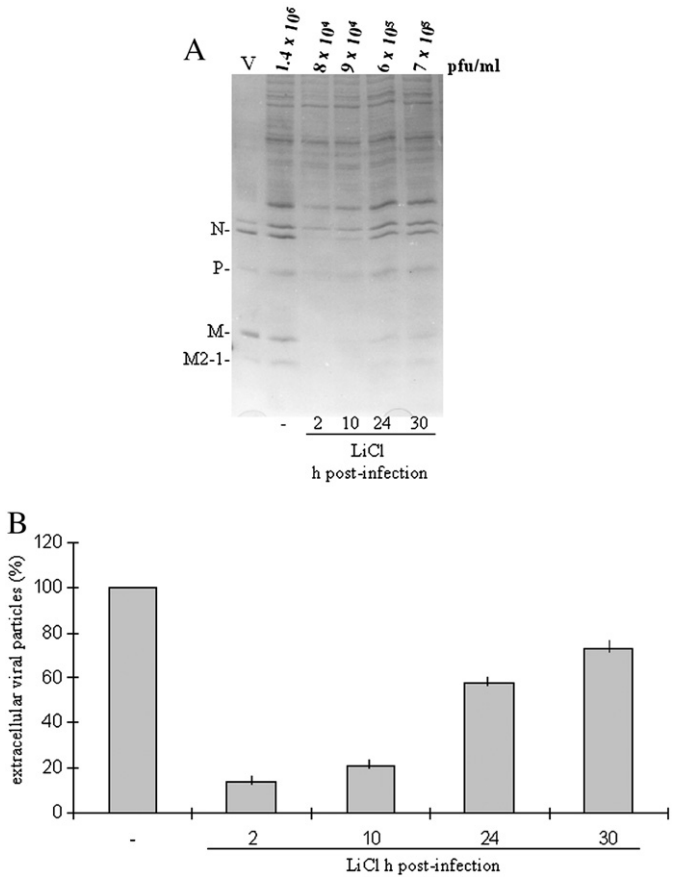

Fig. 4. Effect of lithium addition, at different times post-infection, on the production of extracellular viral particles. (A) Extracellular and infectious viral particles, produced by the HRSV-infected cultures analysed in Figs. 2 and 3, were purified and titered, respectively (see Materials and methods). Their protein composition, after SDS-PAGE and staining with Coomassie blue and infectivities (upper part) are shown. The experiment shown is a representative one of several carried out. V corresponds to purified extracellular viral proteins. (B) Quantitation (by the pCBAS program) of physical extracellular particles produced in three experiments similar to those analyzed in A. The obtained values are expressed as percentages of that obtained for HRSV-infected HEp-2 cells, in the absence of LiCl. Value ranges are indicated.

S54 phosphorylation is removed by the cellular phosphatase PP2A (Asenjo et al., 2005). It is only observed when PP2A is inhibited and it produces a conformational change that decreases P protein electrophoretic mobility, generating the P protein isoform HP (Asenjo et al., 2005).

To determine if P protein was phosphorylated at S54 by the cellular protein kinase GSK-3, P protein was expressed, after transfection or HRSV infection of HEp-2 cells, in the presence of PP2A and GSK-3 inhibitors. SV40 small t antigen (Alberts et al., 1994) and okadaic acid (Bialojan and Takai, 1988) were used to inhibit PP2A in transfected and infected cells, respectively. As GSK-3 inhibitor, LiCl (Stambolic et al., 1996) was used.

The synthetised proteins were labelled with ^{35}S-methionine and from the corresponding cell extracts, P protein was immunoprecipitated, fractionated by SDS-PAGE and visualised by autoradiography. In Fig. 1, panel A, corresponding to transiently expressed P protein in the presence of LiCl or NaCl, HP was only found in the absence of LiCl treatment (NaCl, line), indicating that GSK-3 could be the kinase involved in P protein phosphorylation at S54, when expressed in the absence of the rest of the viral proteins. In Fig. 1, panel B, it is shown that the synthesis and/or accumulation of HP isoform, in HRSV-infected cells, was decreased (about 4 times) by LiCl treatment. Thus, P protein phosphorylation at S54, in the presence or in the absence of the rest of the viral proteins, is controlled by the coordinated action of a cellular lithium-sensitive protein kinase, probably GSK-3 and phosphatase PP2A.

Effect of LiCl on HRSV infection

To determine how the lack of P protein phosphorylation at S54 affected the HRSV growth cycle, we analysed the effect of LiCl on HRSV-infected HEp-2 synthesis and/or accumulation of the following viral products:

Intracellular viral proteins

HEp-2 cells were treated for 48 h with 5, 10, 15, 20 and 50 mM LiCl and the effects of the treatments on cell morphology (observed by phase-contrast microscope), cell viability (determined by permeability to trypan blue) or synthesis/and accumulation of proteins (analysed by SDS-PAGE) were determined (data not shown). Only 50 mM LiCl treatment produced swelling and detachment of HEp-2 cells from the tissue culture plate. At the other lithium concentrations no effects were observed.

The treatment effects of 15 mM LiCl on HRSV-infected HEp-2 cells were determined at 2, 10, 24 and 30 h post-infection. Mock or infected untreated cells (−), were included as controls. Forty eight hours post-infection, the cultures were divided in two parts. From one part, the cells and media were separated and soluble proteins and extracellular viral particles, respectively, were obtained. In Fig. 2, panel A, the protein composition of soluble protein fractions, after SDS-PAGE separation and staining with Coomassie blue is shown. It can be observed that, at early times post-infection, lithium treatment drastically inhibited the synthesis and/or accumulation of viral proteins. Later on only a slight decrease in synthesis and/or accumulation of viral proteins was observed.

In order to quantify the decrease in synthesis and/or accumulation of different viral proteins after 15 mM LiCl treatments, two different amounts of total protein from infected cells were analysed by Western

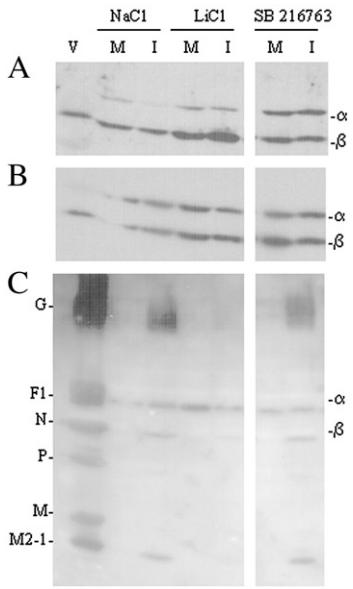

Fig. 5. LiCl and SB 216763 treatments allow GSK-3 phosphorylation. 20 μg of total protein from cellular extracts corresponding to mock (M) or HRSV-infected HEp-2 cells (I) treated with 15 mM NaCl or LiCl or 10 μM SB 216763 from t0 to 48 h post-infection were analysed by Western blot, first with polyclonal antibodies from Cell signalling Cat n° 9331 S that recognised phosphorylated GSK-3 α and β isoforms (A), then with monoclonal antibody Biosource Cat n°44-610 that recognised nonphosphorylated α and β isoforms (B) and then with anti-RS serum (C). V corresponds to purified extracellular viral particles. The electrophoretical mobilities of viral proteins (left) and those of GSK-3 α and β isoforms (right) are indicated.

blot using an anti-RS rabbit serum (Fig. 2, panel B). The quantitation of these data (Fig. 2, panel C), has indicated that the amount of the different viral proteins, was reduced between 97–50% when the lithium-sensitive protein kinase was inhibited early post-infection. At later post-infection times, kinase inhibition resulted in a decrease by 70–30% of different viral proteins, except for N and P proteins whose decrease was only 10%. In both situations, the viral proteins most affected were, in this order: F, M2-1, G and M, while N and P proteins were less affected.

Viral RNAs

Total RNA was obtained from the other half of cultures before analysed. The relative amounts of vRNA (genomic) and cRNA (antigenomic) plus mRNA, were quantified by using specific oligonucleotides of P and M2 genes, first by RT (reverse transcription) and then PCR (polymerase chain reaction), using different amounts of total RNA. The amount of β-actin mRNA was also quantified, in all cases, as an internal control. The results obtained and their quantification are shown in Fig. 3. LiCl treatment of HRSV-infected HEp-2 cells, at early and late post-infection times, inhibited intracellular vRNA by about 90 and 75%, respectively. In contrast, the production of c and mRNA was inhibited by 90%, when the LiCl was added at early post-infection times but not later. Here, the increase was 50%.

Production of infectious and extracellular viral particles

The amount of physical and infectious extracellular viral particles, present in the culture media, were determined after partial purification (Villanueva et al., 1991) and plaque assay, respectively. The results shown in Fig. 4 have indicated that physical and infectious extracellular viral particles were decreased by 85% and 16 fold, respectively, when infected cells were treated with LiCl at early times post-infection. When the treatment was done late post-infection, only 30% and 2 fold reductions respectively, were detected. The difference in decrease of genomic RNA (75%) and that of physical extracellular viral particles (30%) suggests that the morphogenetic process, required to transform RNPs into extracellular viral particles, could be more efficient in the absence of P protein phosphorylation at S54, as anticipated.

The LiCl effect correlates with a decrease in GSK-3 activity

To rule out any effect of LiCl different than that of GSK-3 inhibition, like inhibition of inositol monophosphatase (IMP) (Quiroz et al., 2004) or other protein kinases, HRSV-infected cells were treated, at early times post-infection, with LiCl and 1 mM inositol to revert the inhibitory effect of LiCl on IMP or with SB 216763, another specific inhibitor of GSK-3 (Bain et al., 2007). The results obtained with LiCl and 1 mM inositol were identical to those shown in Figs. 2 and 3 (not shown). With SB 216763, at concentrations between 2 and 60 μM, no inhibition of viral proteins and extracellular particle production was observed (data not shown). It has been indicated that lithium treatment inactivates GSK-3 α and β isoforms, through its phosphorylation at S21 and S9, respectively (Li et al., 2007). However, it is not known if the

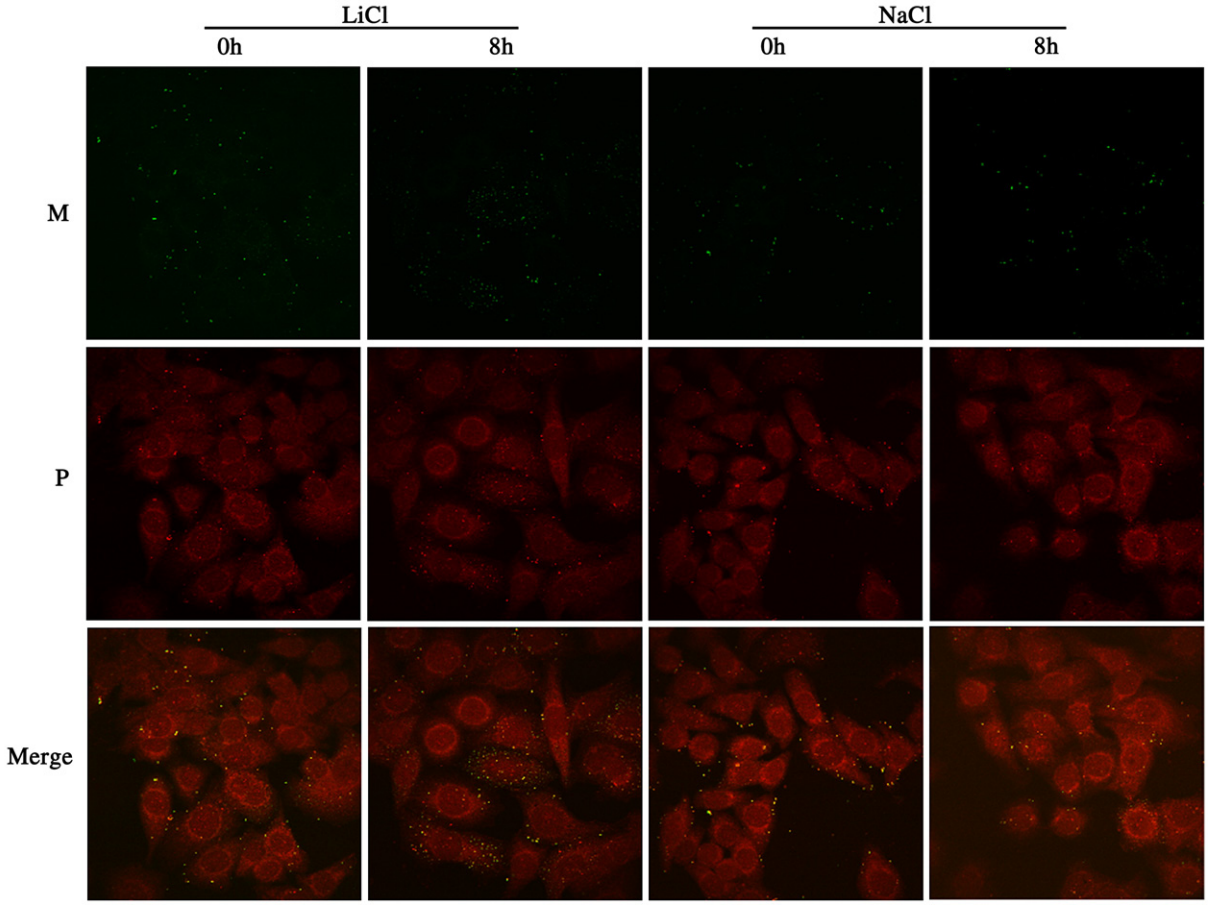

Fig. 6. LiCl treatment of HRSV-infected HEp-2 cells inhibits viral uncoating. Immunofluorescence assays. HEp-2 cells, growing in cover slips, were infected with HRSV Long strain at moi 1. Adsorption, at 4 °C t for 2 h and then infection at 37 °C, were carried out in the presence of 15 mM NaCl or LiCl. At 0, 3 and 8 h post-adsorption, the media was removed and the cover slips were processed as indicated in Materials and methods. The immunofluorescence assays were developed with a monoclonal antibody and a monospecific rabbit serum against M and P protein, respectively, as primary antibodies. As secondary antibodies anti-mouse and anti-rabbit IgG, conjugated with Alexa-488 (green) and Cy-5 (red), respectively, were used. The analyses were done using a Radiance 2100 confocal laser scanning system (Bio Rad Laboratories). Samples corresponding to 0 and 8 h post-adsorption, are shown.

addition of SB 216763 results in a similar effect. Cellular extracts corresponding to HRSV-infected HEp-2 cells treated with NaCl, LiCl or with SB 216763, were analysed by Western blot (Fig. 5) with polyclonal antibodies that recognised GSK-3 α and β isoforms phosphorylated at S21 and S9, respectively (panel A), with a monoclonal antibody that recognises both GSK-3 isoforms (panel B) and with anti-RS serum (panel C). The results have indicated that LiCl addition to HEp-2 cells, allowed a higher phosphorylation on the GSK-3 β isoform than that occurring in the presence of SB 21673 (panel A). Also, it is shown that SB 21675 addition resulted in a significant increase in S21 phosphorylation of GSK-3 α, suggesting a different effect of lithium and SB 21675 on each GSK-3 isoform. The results could indicate that GSK-3 β-isoform is the cellular protein kinase activity involved in P protein phosphorylation at S54, early post-infection. It is noteworthy that GSK-3 β is associated with extracellular viral particles (lane V).

The treatment of HRSV-infected HEp-2 cells with LiCl inhibits viral uncoating

The inhibition of P protein phosphorylation at S54, during HRSV infection, has two main effects. At early times (between 2 and 10 h post-infection), the inhibition of P protein phosphorylation, at this residue, impaired the viral growth cycle, suggesting that early steps of the viral growth cycle are being avoided. By contrast, this P protein phosphorylation inhibition, at later times (between 10 and 30 h post-infection), produced an increase in the amount of viral mRNAs and decrease in that of vRNA. This imbalance has been related to the absence of M2-2 protein activity (Bermingham and Collins, 1999). Since at the beginning of the infection, intracellular viral antigens were poorly detected in the infected cultures treated with LiCl, we reasoned that the early viral growth cycle step, abolished in the absence of P protein phosphorylation at serine residue 54, must occur after adsorption and fusion processes. Thus, that step could be viral uncoating.

Synchronised HRSV-infected HEp-2 cells were treated with 15 mM LiCl or NaCl. At different post-adsorption times, immunofluorescence assays were carried out using a monoclonal mouse specific M protein antibody and a monospecific anti-P rabbit serum. The samples were analysed by confocal immunofluorescence microscopy.

The results in Fig. 6, have shown that both viral antigens were located in punctuated structures (panels M and P). These structures were similar to the intracellular inclusions previously described (Garcia et al., 1993)

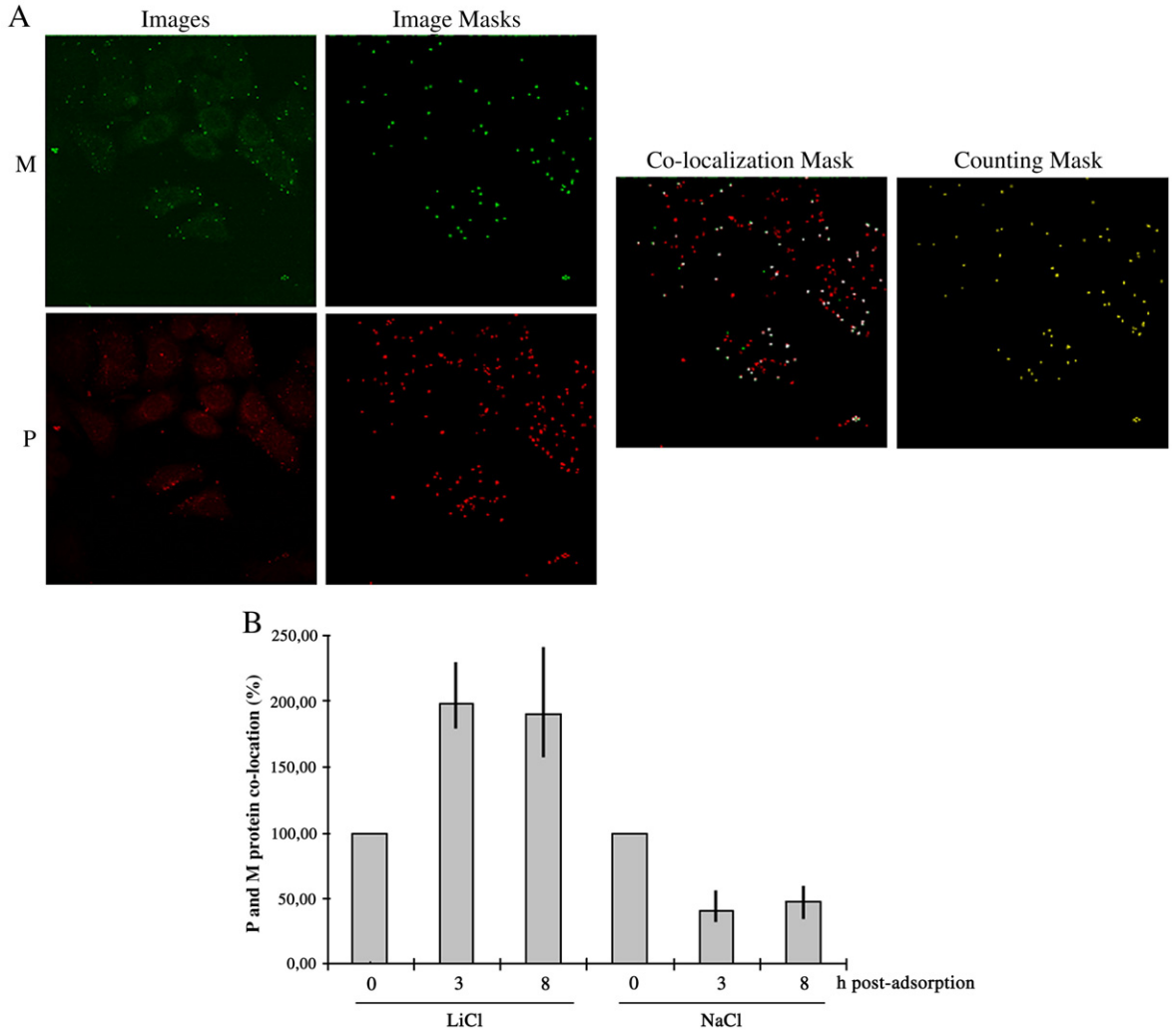

Fig. 7. LiCl treatment of HRSV-infected HEp-2 cells inhibits viral uncoating. Quantitation of M and P proteins co-localised in punctuated structures. Panel A, the first column corresponds to an immunofluorescence assay similar to that shown in Fig. 6 for individual M and P antigens. Column 2 corresponds to individual image masks for each antigen. From them, first a co-location mask and then a counting mask were obtained (right part of the figure), by applying the programs indicated in Materials and methods. Panel B, quantitation corresponding to the results obtained in three different experiments. The values were obtained by applying the analyses shown in panel A to eight different images, corresponding to each experimental condition. Value ranges are indicated by bars. Values are expressed as percentages of those obtained at t0 post-adsorption that was considered as 100%.

and they probably correspond to viral RNPs. In some of these structures both viral antigens co-localised (merge panels), indicating that RNPs were surrounded by M protein. The punctuated structures in which M and P proteins co-localised were counted. An example of that counting, following the procedures indicated in Materials and methods, is shown in Fig. 7A. From immunofluorescence images for M and P proteins, two image masks were obtained, from them a co-localisation mask and from it a counting mask, after elimination of nonco-localised signals. The quantification of the data is indicated in Fig. 7B. Following the adsorption period (t0), the number of punctuated structures in which M and P proteins co-locate was similar in both HRSV-infected HEp-2 cells, treated with NaCl or LiCl. After 3 and 8 h, the number of these structures increased twofold in infected cells treated with LiCl but decreased by half in those treated with NaCl. In these cultures, 24 h post-infection, P protein but not M protein remained in the cytoplasmic inclusions (data not shown), suggesting that P protein phosphorylation at S54 is required for M protein liberation from RNPs of infecting virus.

Effect of 15 mM LiCl treatment in mice intranasally infected with HRSV A2 strain

Three groups of 10 female mice Balb/c were intranasally infected with HRSV A2 strain. At different times post-infection, each group was treated with 15 mM NaCl, 15 mM LiCl or H_2O. Ninety six hours post-infection, the mice were killed and the titres of HRSV in lungs were determined by limiting dilution. The results are shown in Table 1. Among the mice treated with LiCl, 62% of them had no HRSV in their lungs, whereas all those treated with NaCl or H_2O had it. In addition, the lung virus titre of infected mice treated with LiCl, was, at least, 2 log units lower than that found in 37.5% of untreated infected animals. No differences in body weight or fur appearance were observed among the different mouse groups, except for a reduced morbidity in LiCl treated animals. Thus, nasal application of LiCl, after HRSV infection, decreased the amount of infected mice and the amount of infectious virus in their lungs.

Discussion

To develop specific antiviral compounds is important to delimitate genuine viral processes, like RNA synthesis and morphogenesis in Paramyxoviruses. Viral RNPs participate as essential partners in both, without counterparts in humans. Thus, RNP proteins are ideal targets for such compounds without side effects on host cells. To design them, it is important to know how RNP proteins work.

Phosphoprotein P, an RNP component, that forms with L protein the viral RNA polymerase, can make interactions with itself, NC, N0, M2-1 and M proteins, that are essential for viral growth (Khattar et al., 2001; Mallipeddi et al., 1996; Mason et al., 2003; Asenjo et al., 2006). P protein phosphorylations at T108, S116 and T210 are required for viral RNA synthesis, where it acts as a tetramer (Asenjo et al., 2008) whereas modification of P protein at S116, S117, S119 and S232 could have a role in viral morphogenesis (Villanueva et al., 1991; Lu et al., 2002). High turnover phosphorylation at S54, removed by PP2A, inhibits P protein incorporation into M-induced extracellular membranous vesicles (Asenjo et al., 2005).

This paper shows that P protein phosphorylation at S54 was done by a lithium-sensitive kinase, that could be the GSK-3 β isoform. This phosphorylation is required for viral uncoating, the process that liberates infecting viral particle RNPs from M protein, making them active for primary transcription (Lamb and Kolakofsky, 2005). Late post-infection, the lithium-sensitive kinase activity seems to be required for M2-2 protein function.

The GSK-3 action on P protein modification at S54, is supported by the diminished production, in transfected or HRSV-infected HEp-2 cells, of HP isoform (generated by P protein phosphorylation at S54), in the presence of GSK-3 and PP2A inhibitors.

Inhibition of S54 P protein phosphorylation, early after infection, results in a residual viral product production. This suggests a viral infection arrest after the adsorption and fusion processes. In the absence of LiCl sensitive protein kinase activity, viral particles internalise without M protein liberation, suggesting that the early step in the HRSV growth cycle that requires P protein phosphorylation at S54 is that of viral uncoating. In agreement with this, P protein phosphorylation at S54 is never found in extracellular viral particles (Asenjo et al., 2005). The effects on HRSV infection due to GSK-3 activity inhibition, seem to be M protein mediated since no defects on viral RNA synthesis, in a HRSV based minireplicon system, have been detected for P protein variants with substitutions S54D or S54A (Asenjo et al., 2005). These variants simulate full activity or absence of lithium-sensitive kinase, on P protein.

To confirm that GSK-3 inhibition produces the described effects, LiCl action exerted on IMP (Quiroz et al., 2004) was ruled out (by adding LiCl and 1 mM inositol) and SB 216763, another specific GSK-3 inhibitor was tested. No effect was produced by treatment of HRSV-infected cells with different SB 216763 concentrations. In most cells, including HEp-2, there are two GSK-3 isoforms, α and β (Bhat and Budd Haeberlin, 2004). Although both GSK-3 inhibitors used may allow GSK-3 α and ß-isoform inactivation by phosphorylation at S21 and S9, respectively (Li et al., 2007) (Fig. 5), LiCl inactivates the β more than the α isoform, whereas SB 216763 does the opposite (Fig. 5). Therefore, the lithium-sensitive protein kinase, involved in P protein phosphorylation at S54, could be the GSK-3 β isoform. This isoform is present in extracellular viral particles (Fig. 5). This presence could overcome the Akt inhibitory effect on the GSK-3 β isoform (Sun et al., 2008) early post-infection, to avoid cell apoptosis (Peters et al., 2008).

However, other kinases, inhibited by LiCl in vitro, could be involved on S54 P protein phosphorylation, including MPAK-integrating protein kinases (MNK1,MNK2), smooth-muscle myosin light-chain kinase (smMLCK), phosphorylase kinase (PHK), checkpoint kinase (CHK), homeodomain-interacting protein kinase 3 (HIPK3) inhibitory κß kinase and TANk-binding kinase (Bain et al., 2007). However, the presence of all these kinases in HEp-2 cells is uncertain.

HIPK3 is a Fas/FADD-interacting serine/threonine kinase that induces Fas-associated death domain (FADD) phosphorylation, probably at S196 (Rochat-Steiner et al., 2000) and inhibits Fas-mediated Jun-NH_2-terminal kinase activation. FADD primary sequence around this residue is SPMSW, similar to that of P protein S54 (SPITS). Thus, HIPK3 could be also involved in P protein phosphorylation at S54.

LiCl also has an inhibitory effect on mice intranasal infected with HRSV. Intranasal administered, LiCl reduces the number of infected animals, virus titres (Table 1) and morbidity.

Lithium salts have been widely used by ingestion for more than 50 years, for the clinical treatment of human psychiatric disorders

Table 1
Effect of LiCl treatment on HRSV-infected mice. Three groups of 10 female 9 weeks old Balb/c mice were intranasally infected with 50 μl, via their nostrils, of HRSV A2 strain (1×10^8 pfu/ml)

Group	Treatment	Infected mice %	Titre/lung (total pfu)	% of infected mice with each titre
A	LiCl (15 mM)	38	2×10^1–2×10^2	60
			2×10^2–2×10^3	40
B	NaCl (15 mM)	100	2×10^1–2×10^2	62.5
			2×10^2–2×10^3	12.5
			2×10^3–2×10^4	25
C	H_20	100	2×10^2–2×10^3	100

At 4, 10, 24, 32, 48, 52, 72 and 82 h post-infection, each group was treated with 50 μl by nostril of 15 mM LiCl (group A), 15 mM NaCl (group B) or H_2O (group C) and 96 h post-infection the mice were killed. The infectious virus titre in the lungs was determined by limiting dilution. A range of virus titre was deduced (see Materials and methods). The percentage of infected animals in each group (those with detectable virus in their lungs), the titre detected and percentage of infected animals showing different titres, are indicated.

(Cade, 1949; Tondo et al., 1997; Manji and Moore, 1999; Cade, 1999). Therefore, besides its modest reduction in HRSV infections in animals, they could be a safe preventive treatment for acute HRSV infections, especially in patients for whom these infections represent a dangerous risk. It could also be useful to reduce HRSV infections spreading in hospitals, intensive care units and day care centres occupied by babies, toddlers, the immunocompromised and elderly adults.

Materials and methods

Cells and viruses

HEp-2 cells were obtained from the American tissue culture collection. The HRSV Long and A2 strains and the vaccinia recombinant virus vTF-3 were used through this study. The conditions for growing, handling and viral titre determinations were previously described (Asenjo et al., 2006; Villanueva et al., 1991).

Transfection and HRSV infection in HEp-2 cells. Treatments with LiCl and GSK-3 inhibitors. Isotopic radioactive labelling and analysis of synthetised viral proteins. Immunoprecipitation of P protein. Western blot

HEp-2 cells (growing in 8 cm^2 diameter tissue culture plates) were infected vTF-3 at moi 5 and then cotransfected with 2.5 μg of total DNA from pGEM3 recombinant plasmids containing HRSV P gene cDNA and SV40 small t antigen DNA (Asenjo et al., 2005). Ten hours posttransfection the cells were labelled with ^{35}S-methionine 30 μCi/ml; 1000 Ci/mM, for 3 h, in DMEM medium without methionine, containing 15 mM NaCl or LiCl and the corresponding soluble protein fractions were obtained as followed . The cells were scraped separated from media, washed twice with PBS and resuspended in 10 mM Tris–HCl pH 7.5, 140 mM NaCl, EDTA 5 mM, TX-100 1%, DOC 1%. After centrifugation at 12,000 *g* for 15 min at 4 °C, the supernatant was separated and it was considered as the soluble protein fraction (Asenjo et al., 2005).

HEp-2 cells were mock or HRSV Long strain infected at moi 1. After 2 h of adsorption the virus was removed and DMEM medium supplemented with 2.5% inactivated new born calf serum, 2% glutamine and 1% antibiotics. 15 mM LiCl or NaCl or 10–60 μM SB 216763, were added at the indicated post-infection times. Metabolic labelling with ^{35}S-methionine 24 h post-infection, for 3 h, in DMEM without methionine, in the presence of 100 nM okadaic acid (OKA) and preparation of the corresponding soluble protein fractions (48 h post-infection) was as described previously (Asenjo et al., 2005). From the media the extracellular viral particles were partially purified by precipitation twice with 6% polyethylene glycol (PEG) 6000 (Villanueva et al., 1991).

The proteins from the corresponding soluble fractions, were immunoprecipitated with specific P protein monoclonal antibody RS1/P, in the cellular extract buffer containing 0.1% DOC, 0.1% SDS and BSA 0.1 mg/ml. After adding rabbit anti-mouse IgG and *Staphilococcus aureus* Cowan I strain, the immune complexes were recovered under the previously described conditions (Asenjo et al., 2006). They were separated by SDS-PAGE (12% acrylamide) and visualised by Coomassie blue staining or by autoradiography.

To quantify the amount of the different viral proteins synthesised and/or accumulated after treatment with LiCl at different times post-infection, Western blot was developed. Twelve point five, 25 and 50 μg of total protein were separated, transferred to Inmobilon membrane (Villanueva et al., 1991) and incubated with a rabbit serum against HRSV purified extracellular viral particles, as previously described (Rodriguez et al., 2004).

To detect phosphorylated or nonphosphorylated GSK-3 isoforms, polyclonal antibody Cell Signalling Cat n° 9331S and monoclonal Biosource Cat n° 44-610, diluted 1:1000, respectively, were used.

Quantitation of the different viral RNAs synthetised

Total RNA was obtained by Trizol method, 48 post-infection from mock or HRSV-infected HEp-2 cells, untreated or treated with 15 mM LiCl, at the indicated times post-infection, following the suppliers' indications. The different viral RNAs (vRNA, cRNA and mRNAs) were quantified by RT-PCR. Different amounts of total RNA from the different HRSV-infected HEp-2 treated cultures were used for RT-PCR, using the oligonucleotides PmRNA (1–20), PvRNA (622–641), M2mRNA (1–26) and M2vRNA (691–714). The numbers indicate the position of oligonucleotide sequences on the cDNA corresponding to mRNAs of the chosen genes. m and v mind plus and minus polarities, respectively. The RT reaction was carried out using AMVTf and the appropriated oligonucleotide (plus sense to amplify vRNA or minus sense to amplify c and mRNA) following the suppliers' indications, at 42 °C for 45 min, 94 °C for 2 min and 4 °C. Then, the contrary sense oligonucleotide was added together with TfI DNA polymerase. The PCR reaction was 40 cycles at 94 °C for 30 s, 55 °C for 1 min and 68 °C for 2 min. Then, the samples were incubated at 68 °C for 7 min and cooled to 4 °C.

The amplified DNA fragments were analysed by 1% agarose gel electrophoresis in TBE buffer, visualised by staining with ethidium bromide and quantified by a pCBAS program. As an internal control, β-actin mRNA was quantified in a parallel experiment using the specific oligonucleotides and conditions that have been previously described (Martinez et al., 2001). The obtained arbitrary values of amplified DNAs were represented against dilutions of total RNA. The dilutions producing the same amount of amplified DNA were used for the relative determinations of different viral RNA amounts present in each sample.

Immunofluorescence assays

HEp-2 cells growing in cover slips and HRSV Long strain were used for infections. First, adsorption at 4 °C for 2 h, then infection at 37 °C were carried out in the presence of the corresponding salt. At the indicated post-adsorption times the media was removed, the cover slips were washed twice with PBS, and the cells were fixed by treatment with PBS containing 4% formaldehyde, for 10 min at 4 °C. The fixation solution was removed and the cover slips were maintained in PBS containing 2% sodium azide at 4 °C.

The immunofluorescence assays were developed by following previously described conditions (Sullivan et al., 1994). A monoclonal antibody and a monospecific rabbit serum against M and P proteins, at dilutions 1:400 and 1:200, respectively, were used as primary antibodies. The monoclonal antibody anti-M was a generous gift of Dr. Ghildyal. As secondary antibodies anti-mouse and anti-rabbit IgG, conjugated with Alexa-488 (molecular Probes, Eugene, CA) and Cy-5 (Biological Detection systems, Inc., Pittsburgh, P.A), at 1:100 and 1:500 dilutions, respectively, were used. Microscopy was performed using a Radiance 2100 confocal laser scanning system (Bio Rad Laboratories) built on a Nikon Eclipse TE 300 epifluorescence microscope using the 40× oil-immersion planapo objective NA=1.3 and a 1.8× digital zoom. Images were collected at 512×512 pixel (1 pixel=0.33 μm) from a single focal plane (ca.0.64 μm) using a Kalman filter to average three scans at 50 lines per second.

To quantify punctuated structures we used the public domain software imageJv.1.32j (Wayne Rasband, National Institute of Health http://rsb.info.nih.gov/ij/Java1.3.1_03). To each image was applied the *Find edges*, *Threshold* (128–255) and *Analyze Particles* (20 to 200 pixel in size) actions to obtain an image mask. Image masks corresponding to P and M proteins, from the same optical field, were used to obtain a new co-localisation mask using *Co-localisation* plugin. Co-localisation values were obtained applying the *Analyze Particles* action to the co-localisation mask, to render the counting mask.

Mice infection with HRSV and treatment with LiCl

Three groups of 10 female mice Balb/c, 9 weeks old, were infected with 50 μl, via the nostrils, of HRSVA2 strain, containing 1×10^8 pfu/ml. At 4, 10, 24, 32, 48, 72 and 86 h post-infection, each group was treated with 50 μl by the nostrils of 15 mM NaCl, 15 mM LiCl or H_2O. Ninety six hours post-infection, the mice were killed and the HRSV titres in lungs determined by limiting dilution assay. A range of virus titres, expressed as pfu per mouse lung, was obtained by considering the two consecutive dilutions in each case that produced HEp-2 total and partial lysis and the inoculation volume used.

Acknowledgments

We acknowledge Dr. Avila for many suggestions, discussions, critical reading of the manuscript and for providing the GSK-3 inhibitors and antibodies. To E. Langa for developing the panels A and B of Western blot in Fig. 5. To Dr. Ghildyal for her generous gift of anti-M monoclonal antibody. To S. Fernández for her excellent technical assistance. To Dr. I. Outschoorn for English corrections. This work was supported by the intramural projects MPY 1175 and 1276, from the ISCIII and SAF 2002-00070 project from MCyT to N.V.

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